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SITE-SPECIFIC DIFFERENTIATION OF FIBROBLASTS IN NORMAL AND SCLERODERMA SKIN

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14. ABSTRACT

The central hypothesis of this research is that the site-specific differentiation of fibroblasts plays an important role in the anatomic specificity of systemic sclerosis (SSc). During the funding period, (i) we identified appropriate markers and technologies for systematic investigation of the positional identity of fibroblasts, both in normal and diseased tissues. (ii) We found that long noncoding RNAs are the predominant position specific output of the HOX loci, and their misexpression in systemic sclerosis suggests that altered fibroblast positional identity is involved. (iii) Ongoing studies suggest that some long noncoding RNAs are being mis-expressed in activated fibroblasts from SSc.

15. SUBJECT TERMS
Scleroderma, fibroblasts, gene expression

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INTRODUCTION:

In systemic sclerosis (SSc), fibrosis of skin and internal organs occurs in an anatomically reproducible and progressive fashion. The extent and localization of skin involvement are important predictors of long-term outcome and mortality, but the basis of site specificity in SSc is not understood. Fibroblasts are the principal cells in dermis and stroma of epithelial organs that synthesize extracellular matrix proteins, and they are believed to be responsible for excessive fibrosis and tissue hardening in SSc. We recently discovered that fibroblasts are systematically differentiated in a site-specific manner, and they vary significantly in the expression of many genes related to extracellular matrix synthesis and turnover. We hypothesize that the site-specific differentiation of fibroblasts plays an important role in the anatomic specificity of SSc, addressed in three specific aims. At the end of the funding period, we will have characterized fibroblast gene expression patterns in both normal and scleroderma skin and identified the specific fibroblast populations and their gene products that might be associated with SSc progression. This new knowledge will provide basic and much-needed insights on fibroblasts and the pathogenesis of SSc.

Specific Aim 1: Profile fibroblast populations from skin of diverse anatomic sites, thereby creating a reference database of markers for site-specific fibroblast populations.

Specific Aim 2: Construct tissue microarrays of skin and scleroderma tissues to enable high throughput visualization of fibroblast gene and protein expression.

Specific Aim 3: Quantify site-specific populations of fibroblasts in SSc and compare them with site-matched normal and control skin tissues

BODY:

To test the hypothesis that altered fibroblast positional identity may relate to SSc pathogenesis, our first task is to identify reliable markers of fibroblast positional identity in normal skin tissues. In year one of this project, we identified a novel type of genes, termed long noncoding RNAs (lncRNAs), as a major type of transcripts that are expressed in an anatomic specific manner in the body (Rinn et al., 2007). Long noncoding RNAs are transcribed by RNA polymerase II, capped, spliced, and polyadenylated, but do not function by serving as the instruction for protein synthesis. Instead, we and others have found that lncRNAs may serve as the interface between DNA and chromatin modification activities (Amaral et al., 2008; Kapranov et al., 2007; Rinn et al., 2007). The HOX loci encode the master regulators of positional identity in animal cells; the HOX transcription factors are expressed in a nested fashion along the anterior-posterior axis and proximal-distal axis (Wang et al., 2009). We recently characterize the transcriptional landscape of the four human HOX loci at five base pair resolution in 11 anatomic sites (Rinn et al., 2007). We identified 231 HOX ncRNAs that extend known transcribed regions by more than 30 kilobases. HOX ncRNAs are spatially expressed along developmental axes and possess unique sequence motifs, and their expression demarcates broad chromosomal domains of differential histone methylation and RNA polymerase accessibility. Thus, HOX lncRNAs are the predominant position specific output of the HOX loci, and may be ideal biomarkers to address whether positional identity is altered in SSc.

In year two of the project, we have now addressed whether HOX lncRNAs may be misexpressed in SSc lesions. We employed a custom-designed, ultra-high resolution tiling array that interrogated the human HOX loci at five-base resolution (**Figure 1**). We hybridized RNA from site-matched normal skin and lesions of SSc, all derived from the forearm (n=5). We found that nine HOX lncRNAs are consistently dysregulated in SSc skin compared to control skin. Eight of these RNAs are increased in expression; one is decreased in expression. A majority of the lncRNAs increased in expression are those normally expressed in a distal or posterior anatomic fashion. Thus, this preliminary data suggests that accentuation of the distal or posterior fate is associated with SSc. Interestingly, distal anatomic sites, such as fingers, are frequently the initial sites of disease of SSc.

In the second aim of the proposal, we proposed to create tissue microarrays of skin in order to facilitate high throughput interrogation of RNA or protein expression. Gene and tiling microarrays are useful for elucidating the genetic and epigenetic elements that differentiate cell types across the body; however it is equally important to determine the *in vivo* anatomic localization of genes in the three-dimensional context of the skin. To address this challenge, we have constructed a “skin diversity” tissue microarray, where multiple skin sections are placed on a single slide to be used for in situ hybridization and immunohistochemistry. Our tissue microarrays is comprised of 42, two-millimeter formalin-fixed, paraffin-embedded cores of skin from diverse anatomic sites and 8 internal organs such as cervix, intestine, lung, liver and bone (Rinn et al., 2008). Immunohistochemistry or RNA in situ hybridization can be performed on all 50 tissues in parallel, allowing unbiased and high throughput comparison of protein or gene expression levels and localization. A potential limitation of this technology is that proteins and mRNAs present in low levels may be better visualized in frozen sections than formalin-fixed tissues, and conditions for antigen retrieval or signal amplification may need to be developed to visualize low abundance gene products.

To illustrate the use of such a skin diversity tissue microarray, we performed RNA in situ hybridizations for Keratin 14 (K14) and Keratin 9 (K9). As expected, we observed expression of K14 in the basal layer of epidermis in skin from all anatomic sites. K9 is a suprabasal keratin of plamo-plantar skin, and indeed only palmo-plantar skin on our tissue microarray showed strong K9 signal (**Figure 2**)(Rinn et al., 2008). The skin diversity tissue microarray should be useful for the discovery and validation of novel site-specific genes or signaling pathways. Again, this approach can be extended to include tissues of skin diseases to monitor the in vivo expression of genes perturb in disease. The combined power of gene expression, tiling and tissue microarrays will greatly facilitate our understanding of the genes that are important in skin patterning and their roles in skin disease.

In year three of the project, we extended the above results in two ways. First, we tested additional SSc skin samples for altered expression of select HOX lncRNAs. In particular, the expression of one HOX lncRNA, termed nc-HOXC10, is correlated with fibrotic gene expression in fibroblasts. In our initial study, we found nc-HOXC10 level to be elevated in 3 of 5 SSc samples compared to 5 normal skin samples. We tested 6 normal skin samples and 8 SSc skin samples, but found that nc-HOXC10 level was elevated only in one SSc sample. We also examined five samples of idiopathic pulmonary fibrosis and five normal lung samples, and did not see overexpression of nc-HOXC10. Thus in summary, alterations in nc-HOXC10 expression may occur in a subset

of SSc patients, but not in IPF. Second, to determine whether activated fibroblasts in SSc lesions are responsible for lncRNA expression, we analyzed HOX lncRNAs after laser capture of SSc activated fibroblasts vs. non-activated fibroblasts in primary culture. The laser capture microscopy was performed in collaboration with Dr. Francisco Delgaldo's group at University of Leeds. We found several lncRNAs that are consistently increased in expression in SSc activated fibroblasts, including HOTAIR, a lncRNA we recently implicated in enforcing a distal fibroblast-specific chromatin state (Gupta et al.).

KEY RESEARCH ACCOMPLISHMENTS:

- Identification of new position-specific gene markers, including noncoding RNAs
- Interrogation and identification of altered noncoding RNA expression in scleroderma
- Construction of skin tissue microarrays
- Use of skin diversity TMA to interrogate site-specific gene expression

REPORTABLE OUTCOMES:

One publication, listed below.

1. Rinn, J. L., Wang, J. K., Liu, H., Montgomery, K., van de Rijn, M., and Chang, H. Y. (2008). A systems biology approach to anatomic diversity of skin. J Invest Dermatol *128*, 776-782.

Meeting attended:

69th Annual Meeting of the Society for Investigative Dermatology held May 6-9, 2009

Abstract

Programming chromatin states by long noncoding RNAs

The genome is pervasively transcribed, but the function of most RNA transcripts that do not code for protein remains poorly understood. In the course of studying the positional memory of skin, we discovered that one class of cells—the fibroblasts—faithfully retained the anatomic expression pattern of Hox genes from embryonic development through adulthood. The ongoing Hox expression endowed fibroblasts with site-specific inductive activities that control the homeostasis and regeneration of epithelia throughout the body. The epigenetic memory of Hox genes depend on the interplay of Polycomb and Trithorax groups of histone modification enzymes and on long noncoding RNAs (lncRNAs). We discovered 231 candidate lncRNAs in the human Hox loci, which constitutes the predominant anatomic specific output of the Hox loci. One lncRNA, named HOTAIR, binds to and recruits the Polycomb complex to on distant chromosomal loci. These findings suggest that lncRNAs may function as the interface between DNA and chromatin modification machineries, and the ability of lncRNAs to target distant genes highlights possible diverse functions in biology. The connection between perturbation of lncRNAs and human disease will be discussed.

Personnel receiving pay from the research effort

1. Howard Y. Chang, M.D., Ph.D. –Principal Investigator
2. Graciela Enciso—Research Assistant

Additional personnel had other salary support and were not paid from this grant.

CONCLUSION:

These results suggest that one part of the perturbation in scleroderma fibroblasts involves altered positional identity. This alteration is specifically manifested in misexpression of long noncoding RNAs from the HOX loci. Additional samples are necessary to confirm and extend these results.

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Kapranov, P., Cheng, J., Dike, S., Nix, D.A., Duttagupta, R., Willingham, A.T., Stadler, P.F., Hertel, J., Hackermuller, J., Hofacker, I.L.*, et al.* (2007). RNA maps reveal new RNA classes and a possible function for pervasive transcription. Science *316*, 1484-1488.

Rinn, J.L., Kertesz, M., Wang, J.K., Squazzo, S.L., Xu, X., Brugmann, S.A., Goodnough, L.H., Helms, J.A., Farnham, P.J., Segal, E.*, et al.* (2007). Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell *129*, 1311-1323.

Rinn, J.L., Wang, J.K., Liu, H., Montgomery, K., van de Rijn, M., and Chang, H.Y. (2008). A systems biology approach to anatomic diversity of skin. J Invest Dermatol *128*, 776-782.

Wang, K.C., Helms, J.A., and Chang, H.Y. (2009). Regeneration, repair and remembering identity: the three Rs of Hox gene expression. Trends Cell Biol.

APPENDICES:

p. 7-13:

Rinn, J. L., Wang, J. K., Liu, H., Montgomery, K., van de Rijn, M., and Chang, H. Y. (2008). A systems biology approach to anatomic diversity of skin. J Invest Dermatol *128*, 776-782.

SUPPORTING DATA:

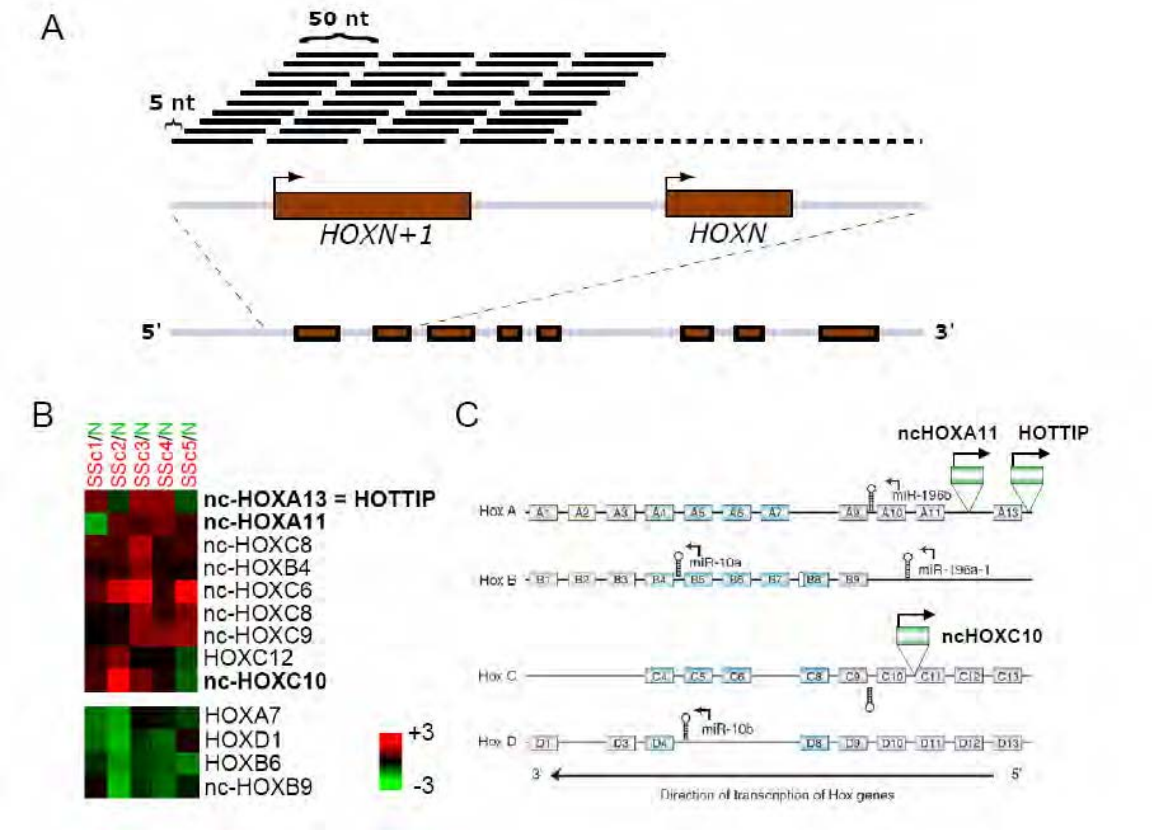

Figure 1. Analysis of HOX ncRNAs in scleroderma. (A) Design of the HOX tiling array. Each probe is a 50 nucleotide (nt) sequence. The probes tile genomic sequences with a 5 base off set across all four of the HOX loci. (B) Identification of specific lincRNAs increased in expression in SSc skin vs. normal site-matched control skin. RNA from SSc samples are labeled with Cy5 dyes (red) and RNA from normal skin samples is labeled with Cy3 (green). These samples are then competitively hybridized to the tiling array. The intensity of the color scale indicates relative increase or decrease of RNA. Each lincRNA is named by the HOX gene 3' to its genomic location. (C) Genomic organization of the HOX loci, highlighting *HOTTIP, ncHOXA11,* and *ncHOXC10*.

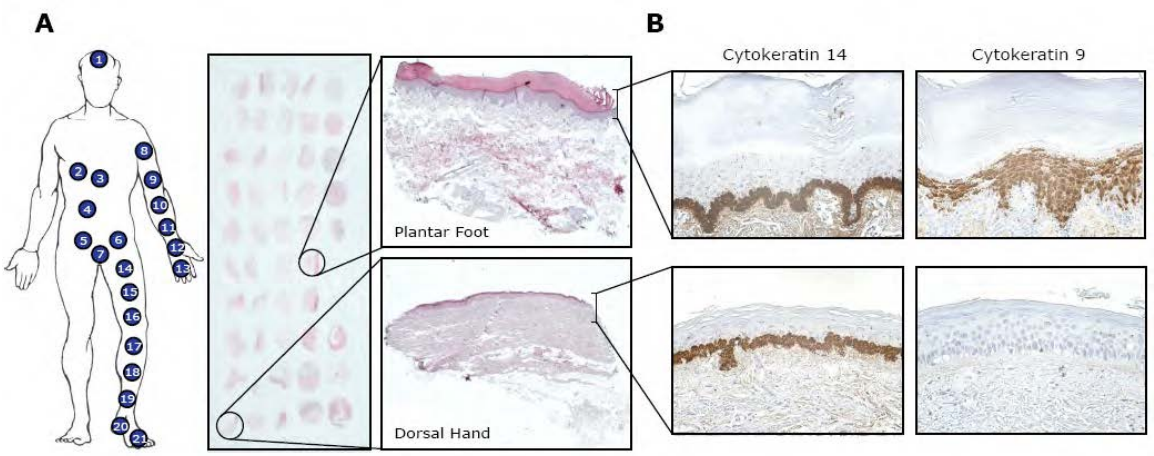

Figure 2. A human skin diversity tissue microarray. (A) Positional map of tissue microarray (left) comprised of 42, two-millimeter longitudinal cross sections of skin and 8 sections of other organs, in total representing 21 unique sites of skin (blue circles). Hematoxylin and Eosin staining of the tissue microarray (middle) and 5X zoom of cores representing plantar (top) and dorsal hand skin (bottom) (B) A 10X zoom of plantar skin section (top) and dorsal hand (bottom) following *in situ* hybridization with probes complimentary of either Keratin 14 or Keratin 9 mRNA.